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Abstract

Transgenesis may allow the generation of farm animals with altered phenotype, animal models for research and animal bioreactors. Although such animals have been produced, the time and expense involved in generating transgenic livestock and then evaluating the transgene expression pattern is very restrictive. If questions about the ability and efficiency of expression could be asked solely *in vitro* rapid progress could be achieved. Unfortunately, experiments addressing transcriptional control *in vitro* have proved unreliable in their ability to indicate whether a transgene will be transcribed or not. However, initial studies suggest that cell culture may be able to predict *in vivo* post-transcriptional events. We review these issues and propose that strategies which engineer the transgene integration site could enhance the probability for efficient expression. This approach has now become feasible with the development of techniques allowing animals to be generated from somatic cells by nuclear transfer. The important step in this procedure is the use of cells grown in culture as the source of genetic information, allowing the selection of specific transgene integration events. This technology which has dramatically increased the potential use of transgenic livestock for both agricultural and biotechnological applications, is based on standard cell culture methodology. We are now at the start of a new era in large animal transgenics.

Introduction

A transgenic animal is one which carries integrated sequences of cloned DNA in its genome. The introduced DNA can be derived from species other than the host and can be modified *in vitro* prior to being introduced into the germline. Therefore, transgenic livestock overcome the limitations of classical animal breeding regimes, where importation of genes by cross-breeding is limited to those traits already present within a given species. Transgenesis may allow the generation of farm animals with altered phenotype (e.g. enhanced growth rate), animal models for research (e.g. to evaluate somatic gene therapy strategies) and animal bioreactors. It is this last opportunity which has seen the most progress over the last decade, with the expression of a variety of proteins having been targeted to the milk of sheep, cattle, pigs, goats and rabbits (Wilmut et al., 1997).

The time and expense involved in generating transgenic livestock and then evaluating the transgene expression pattern is very restrictive. If questions about the ability and efficiency of expression could be asked solely *in vitro* rapid progress could be achieved. In this article, we will describe the various stages at which transgenes can be assessed in cell culture *in vitro* prior to their incorporation into livestock; and discuss the validity and usefulness of these studies for transgenic livestock produced by microinjection and nuclear transfer.

Expression assay systems: cells versus animals

Transcription of eukaryotic genes requires that the gene sequence is present within chromatin and that it has been exposed to the relevant developmental cues. Therefore, different experimental assay systems each have an inherent stringency with regard to identifying transcription control elements. The least stringent assay, transient transfection of cells grown in culture where the introduced plasmid DNA remains

episomal, does not result in the appropriate chromatin structure being formed. The appropriate chromatin structure can be established in stably-transfected cells, where (usually) the introduced plasmid DNA has integrated into the host cell's genome, but the gene does not experience the full repertoire of developmental signals. In transgenic animals, however, both criteria are achieved. The introduced DNA fragment integrates into the host genome and can be inherited in a Mendelian manner. However, gene constructs transferred into the germline using the microinjection method suffer from position-effects (Al-Shawi et al., 1990; Clark et al., 1994).

Position-effects are due to the conformational demands imposed by the chromatin structure at the site of transgene integration, the complexity of the transgene array and the actual sequences present within the transgene. The influence this has on expression can vary considerably; generating no or variegated expression, or resulting in ectopic transgene expression (Farini and Whitelaw, 1995). The expression potential for a given transgene at a given integration site must reside in the chromatin structure the transgene either imposes on itself, regardless of the neighbouring chromatin, or the chromatin structure it finds itself in. As a corollary of this, it is reasonable to propose that strategies which engineer the transgene integration site could enhance the probability for efficient expression.

Determining transcription potential

In transgenic animals, tissue-specific and physiologically regulated expression of the transgene will arise only by including the appropriate genetic elements within its structure. Transcription initiation is a multistep process which involves interactions between the gene proximal basal transcription machinery and more distal elements (Kronberg, 1996). These distal elements are diverse in nature, responding to spatial and temporal stimuli, and include chromatin opening and polymerase modulating activities. Together the transcription complex determines the likelihood of a given gene being expressed. Identification of DNA elements capable of regulating gene expression has relied heavily on transfer of gene fragments into cells grown in culture. However, gene regulatory elements have been identified *in vivo* which were not apparent in cell culture studies (Brinster et al., 1988; compare Burdon et al., 1994 and Webster et al., 1995). Furthermore, a comparison of the effect of various regulatory elements showed examples of high efficiency of expression *in vitro* and only moderately efficient expression *in vivo* (Petitclerc et al., 1995).

These differences can relate to specific transcriptional control elements or present as more generic differences. For example, molecular analysis of the distal enhancer of the mouse α-fetoprotein gene has shown that mutations to specific transcription factor binding sites in the enhancer caused a 10-fold reduction in enhancer function *in vitro*, whereas in transgenic mice, the same mutation resulted in sporadic tissue-specific expression of the transgene, dependent on the site of integration (Millonig et al., 1995). In contrast, mutations to the glucocorticoid receptor element within the ovine β-lactoglobulin (BLG) promoter all but abolished expression in transfected cells but had no effect on expression in transgenic mice, as compared to wild-type constructs (Tom Burdon, personal communication). The same contrariety between *in vitro* and *in vivo* experiments is also observed for the basal transcriptional machinery. Mutagenesis of the αB-crystallin TATA box sequence had no effect on promoter activity in transfected lens cells but preferentially reduced promoter activity in the lens of transgenic mice (Haynes et al., 1997). Therefore, when comparing cell transfection and transgenic mice, it would seem that regulation of transcription differs *in vitro* from *in vivo* at the levels of both the basal transcription machinery and upstream regulatory elements.

There is now considerable evidence to support the hypothesis that a more stringent requirement for introns exists in transgenic mice than in cell culture. For example, whilst introns increase transcriptional efficiency of metallothionein-I promoter driven rat growth hormone gene constructs in transgenic mice, expression in cultured cells seems to be insensitive to the presence of introns (Brinster et al., 1988). Through our study of the ovine BLG gene we have also encountered this phenomenon. Genomic gene fragments of BLG are expressed in a position-dependent manner in the mammary gland of transgenic mice (Whitelaw et al., 1992), while an intronless BLG transgene which retained both 3′- and 5′-flanking sequences was found to be position-dependent. A transgene is said to be expressed in a position-dependent manner when expression is seen in only a proportion of the lines generated: expression is silenced in some lines. We have demonstrated that BLG intron removal *per se* increases the sensitivity of BLG transgenes to position effects *in vivo*, whilst having no effect on expression *in vitro*

(Webster et al., 1997). This suggests that introns play a role in facilitating transcription of microinjected genes and that this effect manifests only on genes exposed to developmental influences, after passing through the germline. There is also the possibility that the differences between *in vivo* and *in vitro* results could reflect the inherent differences between a microinjected linear transgene and supercoiled transfected plasmids or be a property of cell lines *per se*.

It is now becoming apparent that position effects can also result in variable levels of transgene expression, either between individual transgenic animals within a given line or between individual transgenic lines. For example, analysis of transgenic mouse lines carrying the α-globin promoter linked to the lacZ reporter indicated that cells either do not express the transgene or express it at a level characteristic of that line (Robertson et al., 1995). In other words, the number of expressing cells varies greatly between different lines, but there is a similar percentage of expressing cells within the line. Discrete patches of transgene expressing cells surrounded by non-expressing cells have also been observed in the mammary gland of transgenic mouse lines expressing BLG transgenes (Dobie et al., 1996). It has been proposed that variegated silencing may be a consequence of the proximity of the transgene array to centromeric heterochromatin (Dobie et al., 1996) or that multicopy transgene arrays may themselves act as the focus for heterochromatin formation (Dorer et al., 1997).

These expression patterns can be explained by a binary model of expression. This model suggests that a percentage of cells are able to form an active transcription complex and the level of expression is the same in each expressing cell. In the binary model, enhancers increase the number of cells able to undergo transcription by preventing gene repression in individual cells (Walters et al., 1995). In contrast, transgenic mice carrying the β-globin promoter linked to the lacZ reporter showed graded expression on a cell-to-cell basis both within and between transgenic lines (Hammer et al., 1985). The binary effect can be evaluated in cell culture (Walters et al., 1995), however it remains to be determined whether graded expression can be modelled *in vitro*.

Assessment of post-transcriptional events in vitro

Modulation of any of the events between transcription and post-translation modification of the protein could have significant effects on final product levels. This area of gene regulation has not been adequately addressed in transgenic animals: most of the accumulated knowledge on transgene expression concerns transcription potential.

Modulation of the half life and translational potential of mRNA can give rise to large variations in the levels of protein synthesis, independently of any change in transcriptional activity. It is also likely that improper splicing, polyadenylation and nuclear transport or the generation of an unstable cytosolic mRNA will result in the accumulation of low-levels of mRNA, hence reduced protein production. For example, aberrant splicing has been associated with poor expression of human factor IX transgenes in mice. Analysis of the transgene transcript uncovered a cryptic splice site, removal of which resulted in an increase in factor IX production (Yull et al., 1995). A similar situation has been reported for CD46 transgenes (Mulder et al., 1997).

Transcriptional control mechanisms observed *in vivo* are often not consistent with those acting *in vitro*. However, pre-mRNA processing mechanisms observed in cell culture have been shown to correlate with the post-transcriptional modifications observed in transgenic mice. For example, BLG minigene constructs were inefficiently spliced, with the frequency of intron retention similar *in vitro* and *in vivo* (Donofrio et al., 1996). The availability of an *in vitro* system that mirrors RNA processing events *in vivo* is likely to allow testing of transgene constructs for efficient post-transcriptional modifications prior to their use in livestock. As more effort is targeted to addressing post-transcriptional processing events in transgenic animals the validity of cell culture models is gaining strength (Seipelt et al., 1998).

The requirement for introns to achieve optimal mRNA processing is a problem in achieving efficient transgene expression, particularly for large genes. In this context, viral mRNAs, although not containing introns, are efficiently processed. For example, regulatory sequences such as the constitutive transport element of simian retrovirus type-1 augment the translational potential of mRNA transcripts, by commandeering endogenous cellular factors (Saavedra et al., 1997). It is possible that RNA elements could facilitate the translational potential of cDNA based transgenes. These types of constructs can be evaluated *in vitro*.

It may also be possible to modulate post-translational maturation of the transgene product. For example, inefficient endo-proteolytic processing of a

transgene encoded human protein C has been rescued through the co-expression of the processing enzyme furin (Drews et al., 1995). Again, it may be possible to assess protein processing issues in an *in vitro* system.

Nuclear transfer: a new era for cell culture

Transgenic livestock, until recently (Schnieke et al., 1997), were generated by the direct DNA microinjection of fertilised eggs, with the first transgenic livestock produced over a decade ago (Hammer et al., 1985). The microinjection route is inefficient, primarily due to the large number of animals required: 1% of the eggs injected result in a transgenic animal. This approach is also limited by the random nature of transgene integration, with both the site of integration and the number of integrated copies influencing transgene expression (Al-Shawi et al., 1990; Clark et al., 1994; Garrick et al., 1997). In addition, microinjection can only add genes. Taken together, these limitations have been the driving force for the development of a cell-based approach to generating transgenic livestock.

For many years targeted integration has been possible in the mouse model through embryonic stem (ES) cell technology (Thompson et al., 1989). ES cells are unique, in that they allow efficient homologous recombination events to occur between introduced DNA fragments (the transgene) and endogenous genomic loci, while remaining pluripotent. Although there has been considerable effort to generate ruminant ES cells, to date all attempts have been unsuccessful. The application of the recently developed selection protocol for embryonic cells that kills differentiated cells while leaving the undifferentiated (and pluripotent) cells to grow (McWhir et al., 1996), may now provide a route to generate ruminant ES cells. An alternative approach, and one which has generated considerable attention through the generation of Dolly (www.ri.bbsrc.ac.uk), involves the transfer of nuclear genetic material from cells grown in culture into an unfertilised egg cell (Campbell et al., 1996; Wilmut et al., 1997). Now rather than merely evaluating a transgene construct in cell culture, the actual cell is the genetic material from which the transgenic animal is generated. Transgenic livestock have been produced using this technology, e.g. sheep carrying a human factor IX transgene (Schnieke et al., 1997). The gateway has been opened for the production of a variety of livestock for both agricultural and biomedical applications.

There are many advantages to this technology; all founder animals are transgenic, mosaic founders are not produced, the sex of the founder can be selected and a flock/herd of clonal animals can be produced within one generation. More importantly, many aspects of the transgene can be analysed *in vitro* prior to the generation of an animal. The integrity of the transgene locus can be evaluated which may be particularly important when large transgenes, e.g. yACs, are used. Furthermore, with some ingenuity, it will be possible to check the expression potential of a transgene before going through the cost of producing the animal. Expression strategies could, for example, involve the transient expression of the appropriate receptor (Lesueur et al., 1990) or DNA which can mediate trans-induction of expression (Ashe et al., 1997). The most important point, however, is that site-specific integration events through homologous recombination can be selected for, thus allowing strategies involving gene knock-out or replacement to be performed. The mouse model has already shown the feasibility of such strategies for the milk protein α-lactalbumin (Stacey et al., 1995). Future transgenic livestock will probably have the transgene integrated into a permissive site. Although the appropriate genomic site remains to be determined, the approach has been evaluated in the mouse model (Bronson et al., 1996; Melton et al., 1997).

A cell-based method to produce transgenic livestock allows single-copy transgene integrants at predetermined sites to be generated, which should limit problems such as variegated transgene expression (Garrick et al., 1997). The outcome is certainly likely to be more predictable and generate the anticipated transgene expression profile, but will expression levels be high enough? Multiple-copy transgene loci given the right position-effect can give very high-levels of expression. The next generation of transgenes may well have promoters optimised for transcription factor complex formation thereby increasing the probability of transcription and incorporate elements capable of enhancing mRNA processing. It is likely that many of these issues can be evaluated in cells prior to generating a transgenic animal.

Conclusion

The study of gene expression *in vitro* has greatly increased our understanding of transcription and mRNA processing mechanisms and will continue to do so.

It has, however, been limited in its capacity to act as a predictor of how a transgene will function in the whole animal. The recent development of techniques allowing animals to be generated from somatic cells by nuclear transfer is the start of an new era in large animal transgenics. The important step in this procedure is the use of cells grown in culture as the source of genetic information, allowing the selection of specific transgene integration events. This technology which has dramatically increased the potential use of transgenic livestock for both agricultural and biotechnological applications, is based on standard cell culture methodology.

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